^2H and ^{13}C Nuclear Magnetic Resonance Study of *N*-Palmitoylgalactosylsphingosine (Cerebroside)/Cholesterol Bilayers

M. J. Ruocco, D. J. Siminovitch, J. R. Long, S. K. Das Gupta, and R. G. Griffin
Francis Bitter National Magnet Laboratory and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 USA

ABSTRACT ^{13}C- and ^2H-NMR experiments were used to examine the phase behavior and dynamic structure of *N*-palmitoylgalactosylsphingosine (NPGS) (cerebroside) and cholesterol (CHOL) in binary mixtures. ^{13}C spectra of $^{13}C{=}O$-labeled and ^2H spectra of [7,7-$^2H_2$] chain-labeled NPGS as well as 3α-$^2H_1$ CHOL indicate that cerebroside and CHOL are immiscible in binary mixtures at temperatures less than 40°C. In contrast, at 40°C $< T \leq T_c$ (NPGS), up to 50 mol% CHOL can be incorporated into melted cerebroside bilayers. In addition, ^{13}C and ^2H spectra of melted NPGS/CHOL bilayers show a temperature and cholesterol concentration dependence. An analysis of spectra obtained from the melted $^{13}C{=}O$ NPGS bilayer phase suggests that the planar NH-C=O group assumes an orientation tilted 40°-55° down from the bilayer interface. The similarity between the orientation of the amide group relative to the bilayer interface in melted bilayers and in the crystal structure of cerebroside suggests that the overall crystallographic conformation of cerebroside is preserved to a large degree in hydrated bilayers. Variation of temperature from 73°C to 86°C and CHOL concentration from 0 to 51 mol% results in small changes in this general orientation of the amide group. ^2H spectra of chain-labeled NPGS and labeled CHOL in NPGS/CHOL bilayer demonstrate that molecular exchange between the gel and liquid-gel (LG) phases is slow on the ^2H time scale, and this facilitates the simulation of the two component ^2H spectra of [7,7-$^2H_2$]NPGS/CHOL mixtures. Simulation parameters are used to quantitate the fractions of gel and LG cerebroside. The quadrupole splitting of [7,7-$^2H_2$]NPGS/CHOL mixtures and ^2H simulations allows the LG phase bilayer fraction to be characterized as an equimolar mixture of cerebroside and CHOL.

INTRODUCTION

Cerebroside of the central nervous system myelin membrane has been the subject of many biochemical, immunological, and biophysical studies over the past two decades. Fundamental studies characterizing the abundance and type of naturally occurring cerebrosides from normal and pathological mammalian myelin have established that 1) myelin cerebroside occurs almost exclusively as galactocerebroside; 2) galactocerebroside occurs almost exclusively in myelin membrane in a relatively high concentration (~20 weight %); and 3) mammalian central nervous system myelin membrane lipid composition ranges from a 2/1.5/1 to a 2/2/1 (cholesterol/phospholipid/galactocerebroside) mol ratio (Norton and Cammer, 1984; Norton, 1981). Recognition of the preponderance and specific localization of galactocerebroside in myelin stimulated a number of initial biophysical studies of natural lipid extract membranes and monolayers to characterize the thermal behavior (Ladbrooke et al., 1968; Linington and Rumsby, 1981), electric field effects (Clowes et al., 1971), surface potential (Quinn and Sherman, 1971), and surface viscosity (Poss et al., 1979) of galactocerebroside-containing membranes. Crystallographic studies of Abrahamsson et al. (1972) and Pascher and Sundell (1977) elucidated the structure and molecular conformation of cerebroside molecules in crystalline lamellae and showed that intermolecular hydrogen bonds were an important feature of the association between cerebroside molecules. The importance of intra- and intermolecular hydrogen bonding to hydrated cerebroside bilayer membrane stability-metastability was underscored by thermal and x-ray diffraction studies (Ruocco et al., 1981) on homogeneously acylated cerebroside and again by subsequent thermal studies on natural extracted whole fraction, nonhydroxylated and hydroxylated cerebrosides (Curatolo, 1983; Ruocco et al., 1983; Ruocco and Shipley, 1986; Jackson et al., 1988; Johnston and Chapman, 1988). It has been suggested that cerebrosides play a role in regulating the structure and properties of myelin as an insulating membrane (Ruocco and Shipley, 1984; Johnston and Chapman, 1988). Interestingly, neurochemical studies of pathological states of brain and peripheral nervous system myelin (Bologa-Sandru et al., 1981) indicate that cerebroside is a necessary component for normal myelination and nerve impulse conduction.

To better characterize the importance of the molecular structure and mode of intermolecular associations of cerebroside in myelin membrane, systematic thermal (dif-

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Address reprint requests to Dr. Robert G. Griffin, Francis Bitter National Magnet Laboratory and Department of Chemistry, NW-14-5111, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139-4307. Tel.: 617-253-5597; Fax: 617-253-5405; E-mail: griffin@ccnmr.mit.edu.

Dr. Ruocco's present address is Department of Radiology, Walter Reed Army Medical Center, Washington, DC 20307.

Dr. Siminovitch's present address is Department of Physics, The University of Lethbridge, 4401 University Drive, Lethbridge, Alberta T1K 3M4, Canada.

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ferential scanning calorimetry, DSC) and structural (x-ray diffraction and NMR) studies of the phase behavior of galactocerebroside in its pure form and in the presence of other major myelin lipids (phospholipid and cholesterol) have been undertaken (Skarjune and Oldfield, 1979a,b, 1982; Huang et al., 1980; Ruocco et al., 1983; Ruocco and Shipley, 1984; Jackson et al., 1988; Johnston and Chapman, 1988). It was shown that cerebroside exhibits a limited solubility in 1,2-dipalmitoyl-*sn*-phosphatidylcholine (DPPC) gel bilayers (20 mol% *N*-palmitoylgalactosylsphingosine, NPGS) and liquid crystal bilayers at $T \approx T_m$ (DPPC) (Ruocco et al., 1983). Furthermore, even though cholesterol (CHOL) exhibits gel phase immiscibility in cerebroside gel bilayers, the sterol can be incorporated to the extent of 50 mol% into melted liquid crystal NPGS bilayers (Ruocco and Shipley, 1984). In these studies preferential association of cerebroside molecules involving intermolecular hydrogen bonding among the galactosyl headgroups, sphingosine bases, and amide linkages was suggested to explain the observed phase behavior, particularly at low temperatures (Ruocco and Shipley, 1984). These interactions may also affect cerebroside clustering in membranes (Suzuki et al., 1981) and lateral phase separation (Barenholtz et al., 1983). Recent thermal and NMR studies of ceramides interacting with CHOL and phospholipids also indicate they may play a similar role in regulating phase behavior (Florio et al., 1990; Abraham and Downing, 1991; Wiedmann and Salmon, 1991; Hamilton et al., 1993).

Although the structure and phase behavior of cerebroside in natural and model bilayer membranes have been studied extensively, the dynamic properties (e.g., molecular fluctuations and the time scale of such fluctuations) have not been studied in mixed lipid bilayers. Solid-state NMR spectra of specifically labeled lipids allow a detailed examination of the motion and conformation of virtually any position in the molecule. In such a microscopic approach, details of particular molecular events can be associated with thermotropic and/or lyotropic transitions (Wittebort et al., 1981). In general, the NMR technique used in the investigation of labeled lipid compounds has been more sensitive to the onset of thermal phase transitions than DSC; however, to date, the correspondence of NMR data and a macroscopic technique such as DSC has been good (Blume et al., 1982a,b; Blume and Griffin, 1982; Vist and Davis, 1990; Huang et al., 1993).

The unique dynamic characteristics of cerebroside in bilayers were originally reported in a study of chain-labeled cerebroside, *N*-[6,6-$^2H_2$]palmitoylgalactosylsphingosine, by Huang et al. (1980) (see also Griffin, 1981). It was observed that long-axis diffusion of NPGS was slow for $T < T_c$. Further evidence in support of this contention is derived from $^{13}C{=}O$ spectra of [1-^{13}C]NPGS, which show an axially asymmetric rigid lattice powder pattern (Fig. 1 *a*). In addition, ^{2}H-NMR spectra of headgroup-labeled cerebroside have spectral widths of ~100 kHz and demonstrate that the -CHDOH group of the galactose headgroups is restricted in its motion (Huang and Griffin, manuscript to be published). The rigid lattice $^{13}C{=}O$ spectrum and the unusually wide ^{2}H-labeled headgroup spectrum are attributed to the hydrogen bonding capability of the galactose residue and amide group (Huang et al., 1980). The only mode of motion in the acyl chains is then *trans-gauche* isomerization, and this leads to the $\eta \approx 1$ spectra observed for *N*-[6,6-$^2H_2$]NPGS. This behavior is in contrast to that seen in phospholipid spectra where other modes of motion (e.g., molecular long axis rotation) contribute to the observed spectral narrowing (Huang et al., 1993; Blume et al., 1982a,b).

Recently the interactions of CHOL in phospholipid (phosphatidylethanolamine and phosphatidylcholine) bilayers have been extensively characterized by ^{13}C- and ^{2}H-NMR (Blume and Griffin, 1982; Siminovitch et al., 1987; Vist and Davis, 1990; Bonmatin et al., 1990; Huang et al., 1993). ^{13}C- and ^{2}H-NMR spectra were used to define the phase behavior and to distinguish various temperature-composition regions of 1,2-dipalmitoyl-*sn*-phosphatidylethanolamine (DPPE)/CHOL, DPPC/CHOL, 1,2-dihexadecyl-*sn*-phosphatidylcholine/CHOL, 1,2-distearoyl-*sn*-phosphatidylcholine/CHOL, and 1,2-diarachidoyl-*sn*-phosphatidylcholine/CHOL bilayers. Whereas $^{13}C{=}O$ spectra of 2[1-^{13}C]DPPE/CHOL bilayers indicated a region in which there is a coexistence of two conformationally and dynamically inequivalent DPPE molecules, the ^{2}H-NMR spectra of chain and headgroup-labeled DPPE did not clearly exhibit two components. This phenomenon was attributed to the intermolecular exchange of DPPE between gel and "liquid gelatin" bilayer phases in the two-phase composition region. The effect of CHOL on the chain-labeled phospholipid ^{2}H spectra has resulted in a more specific interpretation of the molecular interaction of the sterol with phospholipid (Huang et al., 1993; Blume and Griffin, 1982). Specifically, CHOL is viewed as an intercalating spacer that disrupts the gel bilayer lattice to allow fast axial diffusion (long-axis rotation), and it induces the conformational change in the glycerol backbone region characteristic of the gel → liquid crystal bilayer phase transition. At the same time, the sterol maintains, in large part, the acyl chain conformation characteristic of the gel state (i.e., a high *trans* population) (see Blume et al., 1982a). These effects upon CHOL incorporation into phospholipid bilayers have led to an operational definition of the "liquid gelatin" (LG) phase in which conformational and dynamic properties characteristic of both gel and liquid crystalline phospholipids are present. What distinguishes "liquid gelatin" bilayers from the conventional "melted" liquid crystalline (LC) bilayers observed at high temperatures and/or in pure phospholipid systems is that the former contains greater conformational regularity or order in the acyl chain moieties than the latter. ^{2}H and ^{13}C spectra of the LG and LC bilayers are concurrently axially symmetric because of the presence of rapid axial diffusion, although spectral widths of the LG bilayers are larger. Disordering of the liquid gelatin to

"melted" liquid crystalline bilayers is observed, for example, with increasing temperatures as a decrease in the deuterium quadrupole splitting, $\Delta\nu_Q$, in a sigmoidal fashion (see Blume and Griffin, 1982; Huang et al., 1993). The sigmoidal change in $\Delta\nu_Q$ is associated with the cooperative nature of this transition from the LG to the LC bilayer phase.

Our present study addresses the dynamic and conformational effects of CHOL on a chain-labeled cerebroside, *N*-[7,7-$^2H_2$]palmitoylgalactosylsphingosine, and cerebroside labeled at the amide carbonyl position, *N*[1-^{13}C]palmitoylgalactosylsphingosine. Very different behavior is observed for the cerebroside/CHOL dispersions when compared to that reported for phospholipid/CHOL dispersions (Skarjune and Oldfield, 1979a,b, 1982; Blume and Griffin, 1982; Vist and Davis, 1990; Huang et al., 1993). In particular, two components are present in both ^{13}C- and ^{2}H-NMR spectra. In contradistinction to ^{13}C and ^{2}H spectra of phospholipid/CHOL bilayers, these results indicate that intermolecular exchange between the cerebroside gel and LG phases is slow on both the ^{13}C- and ^{2}H-NMR time scales. This slow intermolecular exchange facilitates both the simulation and quantitation of the ^{2}H-NMR lineshapes and lipid bilayer phases, respectively. Complementary ^{2}H-NMR studies of 3α-$^2H_1$-CHOL-containing cerebroside bilayers are used to corroborate an x-ray diffraction investigation (Ruocco and Shipley, 1984) in which it is indicated that two separate lamellar phases, cholesterol monohydrate and NPGS "crystal" bilayers, may exist at low temperatures (<40°C). Finally, the NMR results are compared and discussed in terms of observed calorimetric and structural behavior. Molecular interpretations of these results are discussed further in light of the molecular composition of myelin membrane.

MATERIALS AND METHODS

Lipid synthesis

[7,7-$^2H_2$]-*N*-Palmitoylgalactosylsphingosine and [1-^{13}C]-*N*-palmitoylgalactosylsphingosine were synthesized using the method of Radin (1972). [7,7-$^2H_2$]Palmitic acid and 1-^{13}C]palmitic acid were synthesized by the methods described by Das Gupta et al. (1982) and Wittebort et al. (1982), respectively. [7,7-$^2H_2$]NPGS and 1-^{13}C]NPGS were purified by silicic acid column chromatography with an elution solvent of $CHCl_3$/MeOH (9/1; v/v) and shown to be chromatographically pure by thin-layer chromatography (TLC), using CH_2Cl_2/MeOH/water (65/25/4; v/v) as elution solvent. The synthesis of 3α-$^2H_1$-CHOL required bromination, CrO_3 oxidation, and zinc debromination of unlabeled CHOL according to Fieser (1983) to form cholest-5-en-3-one. Cholestenone was then reduced with lithium aluminum deuteride in diethyl ether according to the method of Rosenfeld et al. (1954). 3α-$^2H_1$-CHOL was shown to be pure by TLC using an elution solvent. CHOL purchased from Nu-Chek Prep (Elysian, MN) also gave a single spot by TLC.

Sample preparation

Samples consisted of 60–120 mg of lipid. NPGS and CHOL were mixed in dichloromethane and methanol in a small vial, the solvent was evaporated under N_2 at 40°C < T < 50°C, and the final traces of solvent were removed under vacuum for 16–24 h. The lipid mixtures were then transferred to an NMR sample tube (7 mm OD) with a narrow constriction, ^{2}H-depleted water (Aldrich Chemicals, Milwaukee, WI) was added (~70 wt% H_2O), and the tube was flame sealed. Sample equilibrium was achieved by centrifuging the lipid-water sample through the narrow constriction at $T > T_c$ of the lipid mixture (typically 70–80°C). After equilibration, the portion of the NMR sample tube to be placed in the NMR coil was flame-sealed under vacuum. Prepared samples were then heated to $T > T_c$ (NPGS), cooled to room temperature, and equilibrated at room temperature for ≥12 h prior to recording spectra. This protocol is similar to that used in a recent x-ray diffraction and calorimetric study of NPGS/CHOL bilayers (see Ruocco and Shipley, 1984).

NMR spectroscopy

^{2}H- and ^{13}C-NMR spectra were obtained on two different home-built solid-state pulse spectrometers operating at 45.3 MHz for ^{2}H and 79.9 MHz for ^{13}C, respectively. ^{13}C spectra were recorded with Hahn spin-echo or cross-polarization with a 180° refocusing pulse (Pines et al., 1973; Griffin, 1981). The ^{13}C $\pi/2$ pulse was 6.5 μs, and 3-ms mixing times were used for cross-polarization. Full-phase cycling with quadrature detection was used (Griffin, 1981). To minimize radiofrequency heating, low ^{1}H decoupling power and long recycle delays (≥5 s) were used. ^{2}H-NMR spectra were obtained using a two-pulse quadrupole echo sequence, $\pi/2_x$-τ-$\pi/2_y$, with a typical pulse spacing of 50–60 μs. The ^{2}H $\pi/2$ pulse was typically 2.2 to 2.5 μs. Full-phase cycling and quadrature detection were used. Between 500 and 4100 echo signals were accumulated per spectrum. The recycle delay was 0.2 s for all spectra of [7,7-$^2H_2$]NPGS-containing samples; however, delays as long as 7 s were used for spectra of 3α-$^2H_1$-CHOL-containing samples.

RESULTS

The ^{13}C-NMR spectra of [1-^{13}C]NPGS/CHOL mixtures are shown in Fig. 1 as a function of CHOL concentration over the 27°C to 86°C temperature range. The rigid lattice ^{13}C=O spectra of pure NPGS bilayers at temperatures below the gel bilayer → liquid crystal bilayer transition (T_c = 82°C) demonstrate an axially asymmetric lineshape, which is indicative of a rigid, immobile carbonyl group that is situated at the water: headgroup interfacial region of the bilayer (Fig. 1 *a*). These spectra are identical to those obtained for cerebroside bilayers in the absence of water (Griffin, 1981). Above the gel bilayer → liquid crystal transition at 86°C, an axially symmetric spectrum indicative of rapid molecular long-axis diffusion is observed.

Notably, the addition of 10–50 mol% cholesterol results in no significant lineshape or spectral width changes at $T \leq$ 40°C (Fig. 1, *b–f*). In contrast, at higher temperatures (i.e., 60°C ≤ T ≤ 75°C), a second spectral component is observed that corresponds to a melted cerebroside bilayer phase (see Fig. 1). Furthermore, the appearance of this melted component occurs at progressively lower temperatures with increasing CHOL concentrations (compare Fig. 1 *a* with 1 *b–f*).

The coexistence of gel and melted components is conclusively demonstrated by the complementary utilization of ^{13}C cross-polarization and Hahn spin-echo pulse experiments. The ^{13}C=O T_1 for [1-^{13}C]NPGS in the gel phase bilayers is long (>1 min) and necessitates the use of ^{13}C

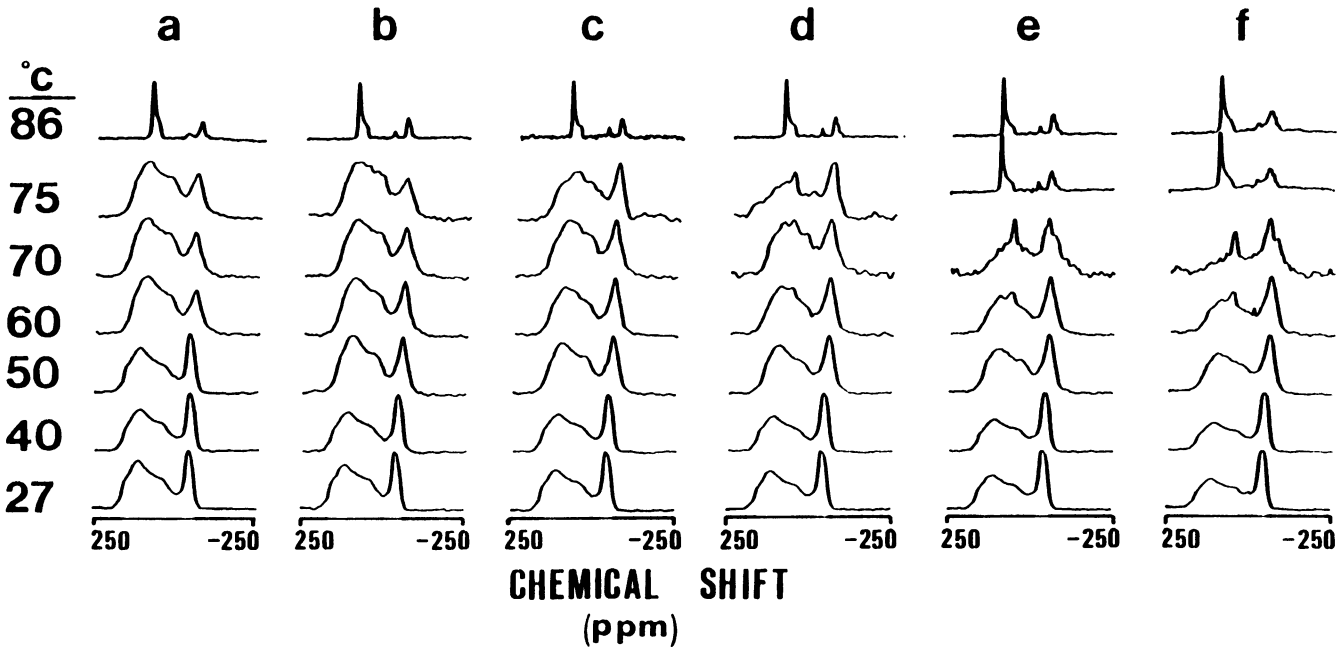

FIGURE 1 Proton-decoupled ^{13}C-NMR spectra of [1-^{13}C]NPGS and [1-^{13}C]NPGS/CHOL mixtures as a function of temperature (°C). (*a*) 0, (*b*) 10, (*c*) 20, (*d*) 30, (*e*) 40, (*f*) 50 mol% CHOL.

cross-polarization experiments to obtain a ^{13}C-NMR spectrum (see Pines et al., 1973; Griffin, 1981). In contrast, [1-^{13}C]NPGS in melted bilayers exhibits a relatively short $^{13}C{=}O$ T_1 and allows the use of a simple Hahn spin-echo with relatively short recycle delay times (RD $\approx$ 5 s). The large difference between the gel ^{13}C T_1 and the liquid crystal ^{13}C T_1 can be exploited in the two-phase region of the [1-^{13}C]NPGS/CHOL bilayer system (as defined by Ruocco and Shipley, 1984) to selectively observe the spectrum of the melted bilayer component. Fig. 2 depicts spectra obtained in the two-phase region (T = 70°C) of NPGS mixtures containing 10–50 mol% CHOL using either a ^{13}C cross-polarization spin-echo that is sensitive to both gel and liquid crystal [1-^{13}C]NPGS components (Fig. 2 *a*), or a spin-echo experiment using a short recycle delay (RD = 5 s $<<<$ T_1(gel[1-^{13}C]NPGS)) that detects only the melted spectral component (Fig. 2 *b*). As would be expected for a given temperature with increasing cholesterol concentration, an increasing proportion of liquid crystal bilayer spectral component is observed. In complementary fashion, the gel spectra component diminishes progressively with increasing cholesterol concentration (compare gel spectral component in Fig. 2 *a* with liquid crystal spectra in Fig. 2, *a* and *b*).

The study of the linewidth dependence of the axially symmetric ^{13}C powder spectra of the NPGS/CHOL liquid crystal bilayers is facilitated by the tendency of cerebroside and cerebroside/CHOL bilayers to supercool to approximately 73°C (Ruocco et al., 1981, 1983). In Fig. 3 we show the axially symmetric spectra of melted cerebroside bilayers containing 0, 30, and 50 mol% CHOL. The residual chemical shift anisotropy, $\Delta\sigma_R$ (where $\Delta\sigma_R = \sigma_\parallel - \sigma$), is plotted as a function of temperature at a constant CHOL concentration in Fig. 4. For a given CHOL concentration the chemical shielding anisotropy decreases as a function of increasing temperature. However, for a given temperature it increases with increasing CHOL concentration.

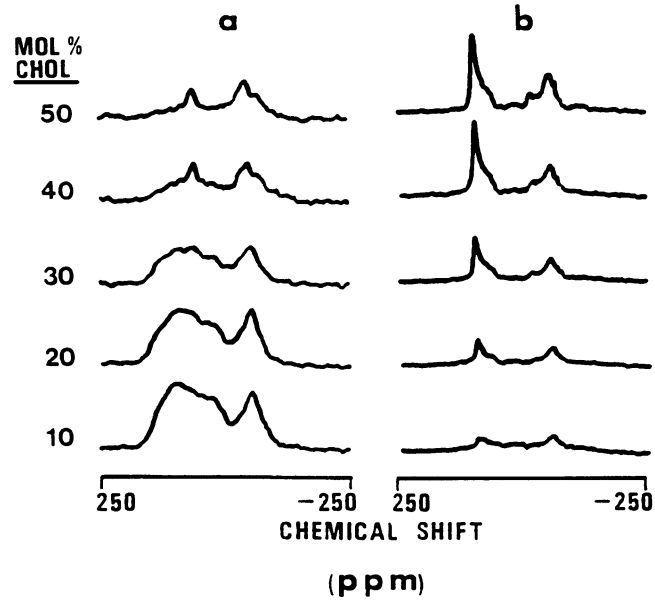

FIGURE 2 Proton decoupled ^{13}C-NMR spectra of [1-^{13}C]NPGS/CHOL mixtures (T = 70°C) using (*a*) cross-polarization with a refocusing pulse or (*b*) simple Hahn echo pulse sequence.

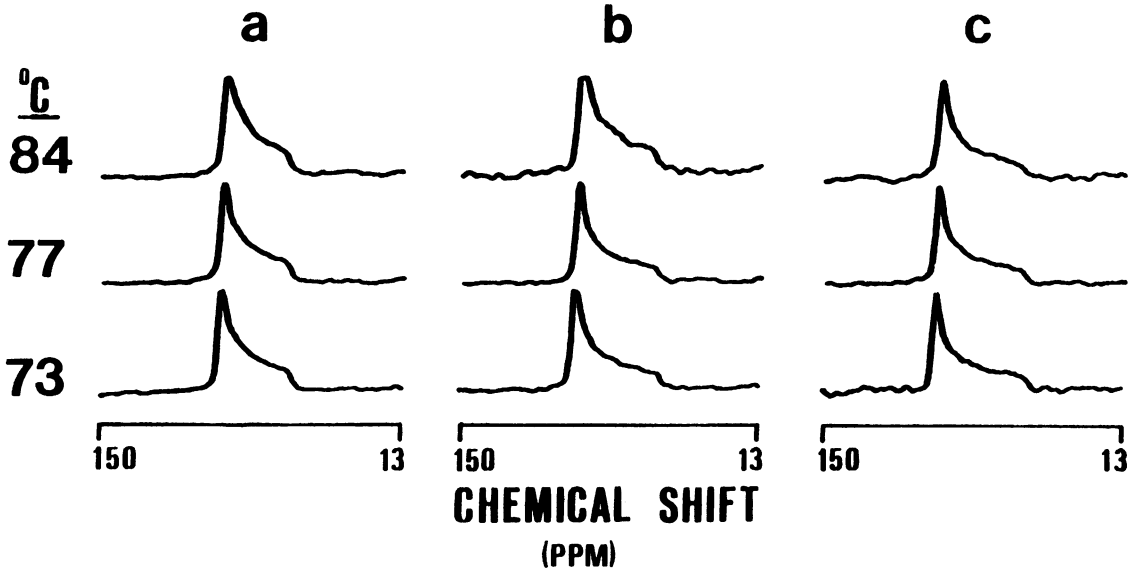

FIGURE 3 Proton-decoupled ^{13}C-NMR spectra of [1-^{13}C]NPGS/CHOL bilayers as a function of temperature in the melted state. (*a*) 0, (*b*) 30, (*c*) 50 mol % CHOL.

Fig. 5 shows the ^{2}H-NMR spectra of [7,7-$^2H_2$]NPGS in bilayers containing 0–50 mol% CHOL as a function of temperature. In pure NPGS bilayers, ^{2}H-NMR lineshapes similar to those reported previously by Huang et al. (1980) are observed. However, the NPGS/CHOL spectra in Fig. 5 *b* through 5 *e* at 50°C $\leq T <$ 86°C are quite different from ^{2}H-NMR spectra obtained for deuterium chain-labeled phospholipid/CHOL bilayers at similar temperatures (i.e., $T < T_c$) (e.g., see Skarjune and Oldfield, 1979a,b, 1982; Huang et al., 1993). In particular, for temperatures below T_c of NPGS, the spectra are a superposition of NPGS gel and melted NPGS liquid crystal spectral lineshapes, indicating that exchange between the liquid crystal and gel phases is slow on the ^{2}H-NMR time scale. The residual quadrupole splittings, $\Delta\nu_Q$, of the axially symmetric liquid gelatin (liquid crystal) spectral component are plotted as a function of temperature at a constant CHOL concentration (Fig. 6 *a*). Interestingly, the $\Delta\nu_Q$ is independent of CHOL concentration in a limited temperature range (50°C $\leq T \leq$ 65°C), beyond which the $\Delta\nu_Q$ disperse as a function of CHOL. For example, the increase in magnitude of the quadrupole splitting as a function of CHOL concentration is readily apparent near the transition temperature of NPGS (i.e., 80 to 86°C). Fig. 6 *b* better illustrates that $\Delta\nu_Q$ is constant over the 10–50 mol% CHOL concentration range between 50°C and 65°C. Changes in $\Delta\nu_Q$ as a function of CHOL at a given temperature are observed at temperatures of ≥65°C and are further accentuated at higher temperatures (see T = 70° to 86°C in Fig. 6 *b*). A second notable feature in Fig. 6, *a* and *b*, is the magnitude of the $\Delta\nu_Q$ in the presence of CHOL at 50°C $\leq T \leq$ 65°C ($\Delta\nu_Q$ = 50–54 kHz), which indicates spectral widths, Δ, where 96 $\leq \Delta <$ 102 kHz, similar to or slightly less than that observed for cerebroside in gel bilayers without cholesterol (i.e., $\Delta \approx$ 125 kHz). As pointed out previously, these spectral parameters are characteristic of liquid gelatin bilayers (see Huang et al., 1993).

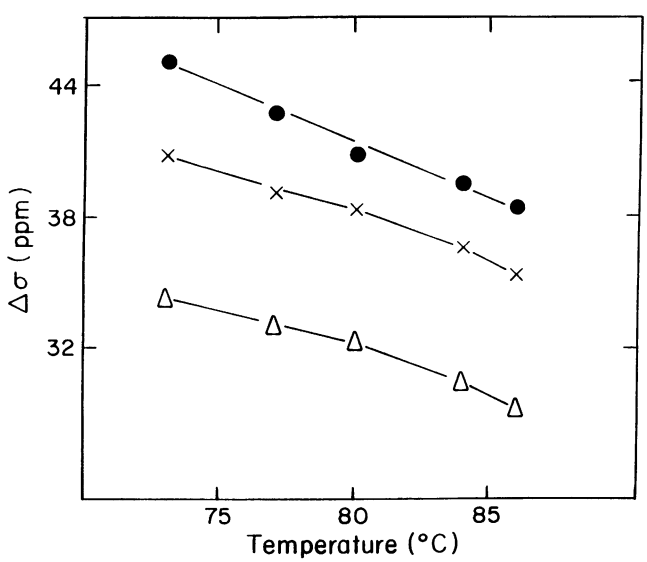

FIGURE 4 Chemical shift anisotropy, $\Delta\sigma$, for [1-^{13}C]NPGS/CHOL melted bilayers (see also Fig. 3) as a function of temperature and CHOL concentration. (△) 0, (×) 30, (●) 50 mol % CHOL.

The spectra and phase behavior of deuterium-labeled 3α-$^2H_1$-CHOL in NPGS bilayers are summarized in Fig. 7. The NPGS 3α-$^2H_1$-CHOL (50 mol%) mixture was prepared as described by Ruocco and Shipley (1984). In brief, the dispersion was equilibrated in the presence of deuterium-depleted water at 70° to 86°C, slowly cooled to room temperature (~25°C), and equilibrated for ≥12 h at this temperature. The ^{2}H-NMR lineshape observed at 19.5°C after this particular regimen is depicted in Fig. 7 *b*. This

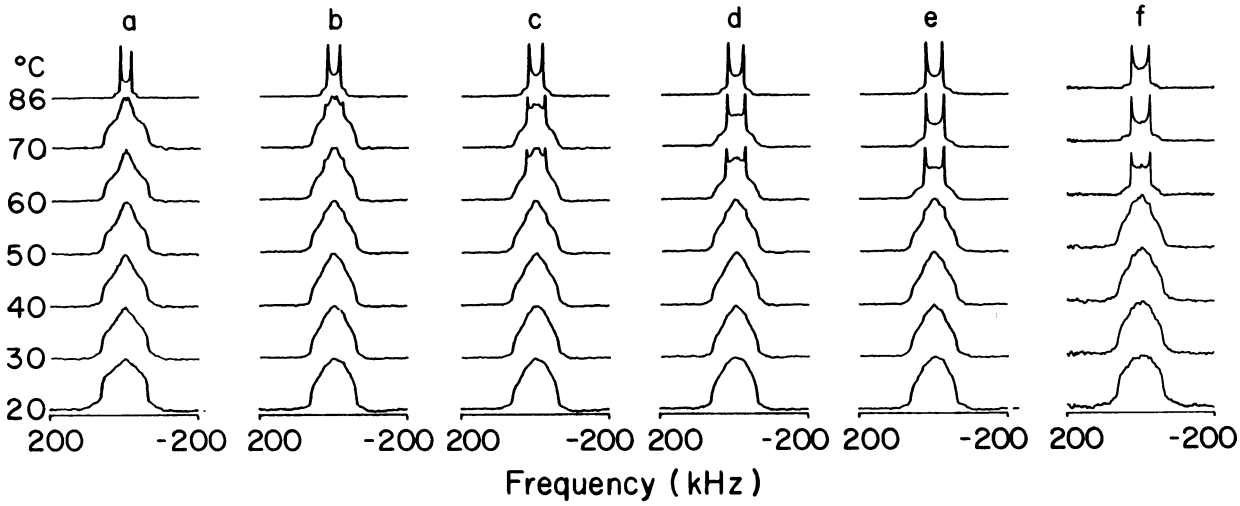

FIGURE 5 ^{2}H-NMR spectra of [7,7-$^2H_2$]NPGS as a function of temperature. (*a*) 0, (*b*) 10, (*c*) 20, (*d*) 30, (*e*) 40, (*f*) 50 mol % CHOL.

lineshape exhibits two sharp peaks corresponding to the perpendicular edges of a rigid lattice spectrum ($\Delta\nu_Q \cong 122$ kHz). The spectral width and lineshape in Fig. 7 *b* for the hydrated NPGS/3α-$^2H_1$-CHOL dispersion are identical to that observed for dry NPGS/3α-$^2H_1$-CHOL powder at 19.5°C (compare Fig. 7 *a*), with the exception that in Fig. 7 *b*, a pair of sharp spectral features at ±22.5 kHz and a central resonance due to ^2H_2O are observed. The rigid lattice spectra observed in Fig. 7, *a* and *b*, are identical to that observed for "dry" crystalline cholesterol at 19.5°C

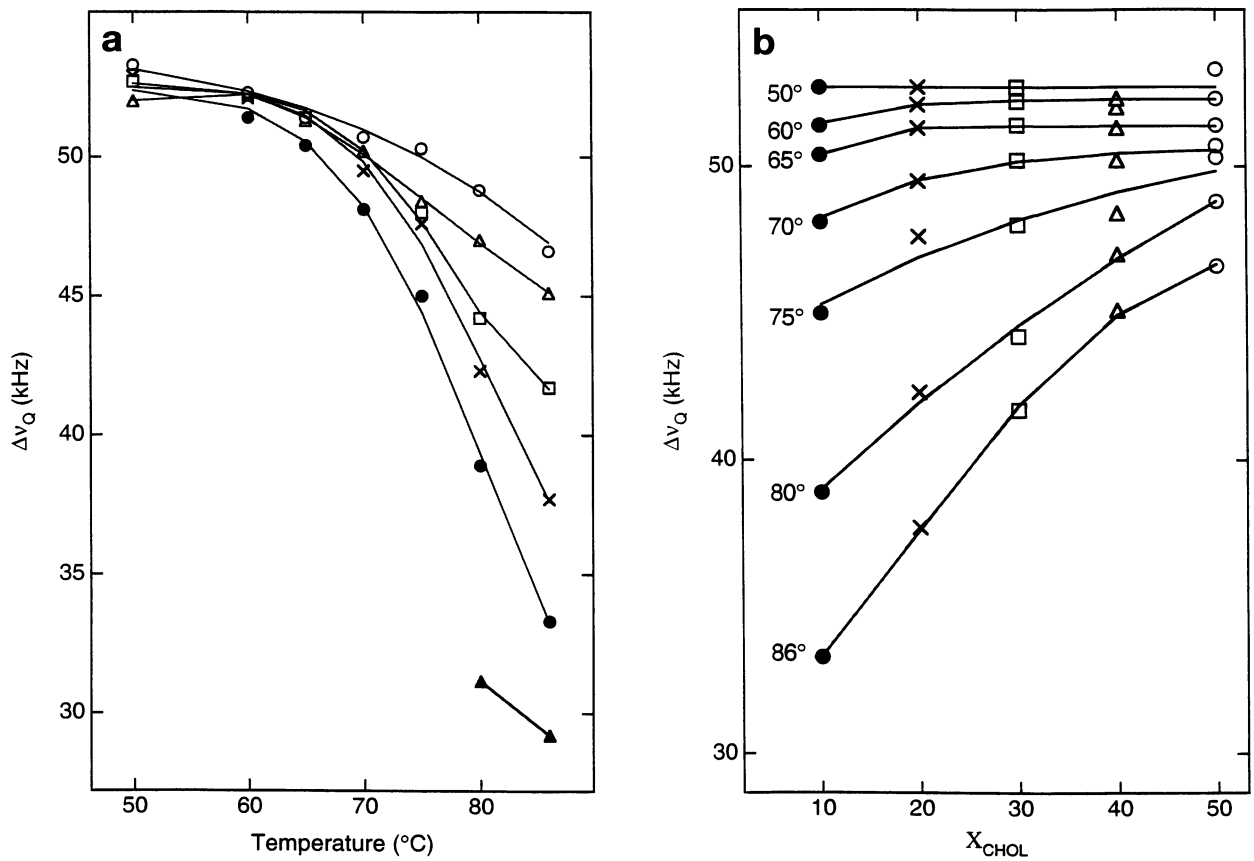

FIGURE 6 Residual quadrupole splitting, $\Delta\nu_Q$, for [7,7-$^2H_2$]NPGS/CHOL mixtures. (*a*) Versus temperature (▲) 0; (●) 10, (×) 20, (□) 30, (△) 40, and (○) 50 mol% CHOL. (*b*) Versus CHOL concentration.

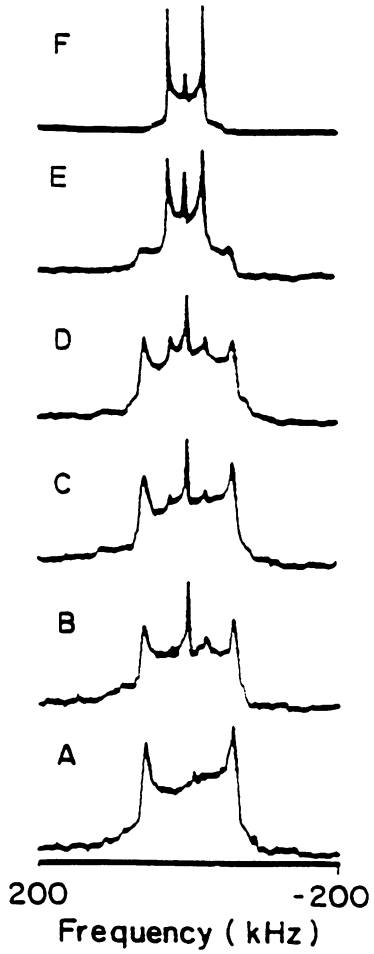

FIGURE 7 ^{2}H-NMR spectra of NPGS/3α-$^2H_1$-CHOL (50/50 mol ratio) dispersion as a function of temperature. (*a*) 19.5°C, dry powder; (*b*) 19.5°C, hydrated dispersion; (*c*) 39.5°C, hydrated dispersion; (*d*) 49.5°C, hydrated dispersion; (*e*) 53°C, hydrated dispersion; (*f*) 66°C, hydrated dispersion.

(Ruocco and Griffin, unpublished data). (The term "dry" is used because precautions to monitor the formation of cholesterol monohydrate were not taken (see Loomis et al., 1979). Nevertheless, identical lineshapes were observed for 3α-$^2H_1$-CHOL in H_2O and "dry" 3α-$^2H_1$-CHOL at 19.5°C, indicating that the state of hydration of CHOL crystals has no significant effect on the spectral lineshape (Ruocco and Griffin, unpublished results).)

As the temperature is increased from 19.5°C to 49.5°C, the central peaks at ± 22.5 kHz are enhanced relative to the rigid lattice spectral component (see Fig. 7, *c* and *d*). At still higher temperatures (see T = 63°C, Fig. 7 *e*), a motionally narrowed component with $\Delta\nu_Q$ = 46.8 kHz dominates the spectrum until T > 56°C, where a single, motionally narrowed, axially symmetric powder pattern is observed (see Fig. 7 *f*; T = 66°C). The spectrum depicted in Fig. 7 *f* is characteristic of CHOL that has been totally incorporated into the melted liquid crystal cerebroside bilayers.

X-ray diffraction experiments indicate that CHOL remains completely incorporated into the liquid-gel phase cerebroside bilayers only at temperatures of ≥56°C. For T < ~56°C (transition I), the sterol gradually separates from the cerebroside bilayer to form a cholesterol monohydrate lamellar phase (Ruocco and Shipley, 1984). (NPGS/CHOL lamellar dispersions exhibit complicated thermal behavior in which two main order-disorder thermotropic transitions occur over the 20–90°C temperature range. Transition I is centered at approximately 50–60°C for dispersions containing ≤50 mol% CHOL. Transition II corresponds to the pure NPGS gel liquid crystal transition (e.g., see figure 10 of Ruocco and Shipley, 1984).) In the NMR experiment, this phase separation process can again be studied by taking advantage of the large difference in T_1 between cholesterol in melted bilayers and in cholesterol monohydrate crystals. With a short (0.2 s) recycle delay, one selectively monitors the motionally averaged ^{2}H signal arising from cholesterol incorporated in the LG phase cerebroside bilayer (see Fig. 8). However, when the cholesterol monohydrate lamellae form, the sterol exhibits a long T_1 characteristic of rigid crystalline solids, and spectra of this species necessitate a much longer recycle delay time (7 s). As shown in Fig. 8, the spectral intensity decreases as the cerebroside/CHOL bilayers are cooled below transition I, until at T = 41°C essentially all but a residual intensity due to cholesterol remaining in cerebroside bilayers is observed. By simply lengthening the recycle delay to ~7 s, following equilibration conditions as described in Materials and Methods, one can obtain the broad, axially symmetric rigid lattice spectrum, as observed in Fig. 7 *b*.

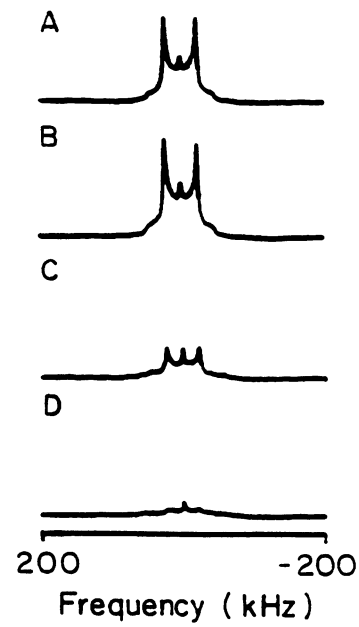

FIGURE 8 ^{2}H-NMR spectra of cerebroside/3α-$^2H_1$-CHOL (50/50 mol ratio) dispersion upon cooling from 66°C. (*a*) 66; (*b*) 56; (*c*) 46; (*d*) 41°C, respectively.

DISCUSSION

^{13}C spectra of NPGS/CHOL mixtures

The ^{13}C spectra of NPGS gel bilayers at 25°C yield an axially asymmetric powder pattern of spectral width ~130 ppm, which is indicative of a rigid, relatively immobile $^{13}C{=}O$ chemical shift tensor on the ^{13}C-NMR time scale ($<10^{-3}$ s). On a molecular level the persistance of this spectrum at higher temperatures and with increasing amounts of CHOL suggests the presence of headgroup and/or amide group inter- and intramolecular hydrogen bonding. As a result of these intimate, specifically directed molecular associations, two separate lipid phases, NPGS "crystal" bilayers and cholesterol monohydrate lamellae, are observed by x-ray diffraction at low temperatures (e.g., $T = 20$°C) (Ruocco and Shipley, 1984). Because these two phases are noninteracting, relatively little change in the gel ^{13}C spectra of NPGS/CHOL dispersions (compared to pure NPGS dispersions) is observed at low temperatures (see Fig. 1, *a–f*; $T = 27$°C).

At intermediate temperatures (i.e., 40°C $\leq T <$ 70°C) a second spectral component arises that, under all conditions used for the cross-polarization pulse sequence, gives rise to a distorted signal corresponding to a melted NPGS/CHOL liquid gelatin fraction superimposed on the rigid ^{13}C gel spectrum (see Fig. 1, *d–f*; $T = 70$°C and Fig. 2, *c–e*, for example). The liquid gelatin component, which is observed in the two-phase region through the use of the simple spin echo, arises because of the incorporation of cholesterol into the cerebroside bilayer (vide infra). The incorporation of steroid disrupts the highly ordered, two-dimensional lattice of the cerebroside bilayer, allowing axial diffusion of the cerebroside molecules, which leads to the fast limit, motionally averaged, axially symmetric ^{13}C powder patterns (see Fig. 2 *b*). The appearance of the rotationally averaged spectral component for all CHOL-containing mixtures at $T \geq 60$°C corresponds to the occurrence of transition I, as described in detail in a calorimetric study of NPGS/CHOL dispersions (Ruocco and Shipley, 1984).

At temperatures beyond transition II for each mixture, a single-component, axially symmetrical ^{13}C powder pattern is observed. There are two interesting features to note concerning the spectra shown in Fig. 1 (*a–f*), $T = 86$°C, and Fig. 2. First, the spectra are not collapsed narrow lines as described for *sn*-2[1-^{13}C]acyl-labeled phospholipids studied to date (see Wittebort et al., 1981, 1982; Blume et al., 1982a,b; Blume and Griffin, 1982; Lewis et al., 1984). In the case of DPPE, 1,2-dimyristoyl-*sn*-phosphatidylcholine, 1,2-distearoyl-*sn*-phosphatidylcholine, 1-myristoyl, 2-palmitoyl-*sn*-phosphatidylcholine, and 1-myristoyl, 2-stearoyl-*sn*-phosphatidylcholine narrow *sn*-2 $^{13}C{=}O$ spectra characteristic of the melted L_α-bilayer phase are observed. This narrow spectrum has been discussed in detail and shown to result from rapid long-axis diffusion of a carbonyl tensor that has its unique axis, $\sigma_\parallel$, oriented at or near the "magic angle" ($\beta = 54.7$°) with respect to the axis of motional averaging (i.e., the molecular long axis) (Wittebort et al., 1981, 1982). Because the ^{13}C and ^{2}H spectra of [1-^{13}C]NPGS/CHOL bilayers are in the fast limit regime (see above and below), the breadth of the powder pattern depends on the orientation of the carbonyl group with respect to the molecular long axis. Second, the breadth of the melted LG and/or LC single-component spectra (Fig. 1, *a–f*, $T = 86$°C, and Fig. 4) varies between ~5 and ~10 ppm as a function of temperature and CHOL concentration, respectively. Again, because we observe fast limit spectra, the variation of the LC chemical shift anisotropy with temperature and composition results primarily from minor changes in orientation of the amide carbonyl group. Spectral broadening of the $^{13}C{=}O$ spectrum has also been observed for phospholipid/CHOL LG/LC bilayer phases (Wittebort et al., 1982), although not to the extent observed for cerebroside/CHOL bilayers.

The conformation space of the amide carbonyl group of palmitoylcerebroside in the liquid crystal bilayer phase can be determined from the expression for rapid rotational averaging of the chemical shielding tensor. The breadth of a motionally averaged tensor, $\Delta\sigma_R$, is calculated using

$$\Delta\sigma_R = \left[\sigma_{33} - \frac{1}{2}(\sigma_{11} + \sigma_{22})\right] \cdot \left[\frac{1}{2}(3\cos^2\beta - 1)\right] + \frac{3}{4}(\sigma_{11} - \sigma_{22})\sin^2\beta\cos 2\alpha, \quad (1)$$

where σ_{ii} values are the rigid lattice tensor elements, and α and β are Euler angles that specify the tensor orientation relative to the director (i.e., the axis about which the motional averaging occurs). Note that α and β correspond to the azimuthal and polar angles, respectively. For an axially symmetrical rigid lattice tensor, the second term in Eq. 1 vanishes and therefore a measurement of $\Delta\sigma_R$ leads to a single value of β. However, for an axially asymmetric rigid lattice tensor, a given $\Delta\sigma_R$ will be consistent with a range of Euler angles α and β. Nevertheless, it is possible to exclude a large range of orientations. The conformation space for NPGS in the absence and presence of CHOL in liquid crystal bilayers was calculated by assuming that the principal axes of the chemical shift tensor of the amide carbonyl have the same orientations as found in the dipeptide crystal, GlyGly · HCl · H_2O (Stark et al., 1983). The principal component, σ_{33}, is orthogonal to the amide plane in which the coplanar principal components σ_{22} and σ_{11} form angles of 13° and 77° to the C=O bond, respectively (see Fig. 9 *a*). Using the principal values of the rigid lattice chemical shift tensor obtained from [1-^{13}C]NPGS, the $\Delta\sigma_R$ (see Fig. 4) has been calculated by covarying the Euler angles α and β. The range of α's and β's that yield the experimental chemical shift anisotropy are listed in Table 1 for pure NPGS and equimolar NPGS/CHOL liquid crystal bilayers at $T = 73$°C and 86°C. Although β is constrained to a relatively narrow range of angles (≤20°), the value of α is considerably less restricted. This type of behavior has

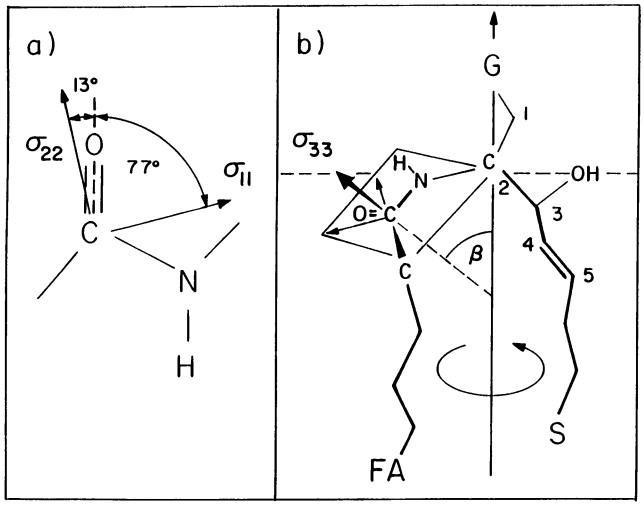

FIGURE 9 (*a*) Orientation of principal components of C=O chemical shielding tensor in the amide group of Gly · Gly · HCl · H_2O (from Stark et al., 1983). Note that σ_{33} is orthogonal to the amide plane. (*b*) Proposed conformation of amide group in palmitoylcerebroside. Principal components of chemical shielding tensor are shown in amide plane. The amide plane contains carbon 2 of both the sphingosine base and acyl chain. The angle β is described in the text. G, galactose headgroup; F, acyl chain; S, sphingosine base. Bilayer interfacial plane (*dashed line*) is shown normal to the molecular long axis (*arrow*). Sphingoid chain carbons are numbered.

been observed previously (Skarjune and Oldfield, 1979a,b, 1982).

For pure NPGS liquid crystal bilayers, β ranges from 38° to 55° (Table 1). Because β is analogous to the polar angle θ, the results indicate that σ_{33}, the principal tensor component orthogonal to the amide plane, is between 38° and 55° from the director axis at T = 86°C. Assuming the director axis is coincident with the molecular long (chain) axis and, furthermore, that this axis is orthogonal to the bilayer interface in the L_α phase (Tardieu et al., 1973; Luzzati and Tardieu, 1974), the average conformation shown in Fig. 9 *b* is proposed. The striking feature of this conformation is that the amide plane lies at an inclination to the bilayer interface rather than parallel to the surface. This inclination is between 35° and 52° with respect to the bilayer surface. Interestingly, the amide plane in the crystal structure of hydroxystearoylcerebroside (Pascher and Sundell, 1977) is inclined at 58° with respect to the director axis. The director in the crystallized form of cerebroside is described by a line parallel to the acyl and sphingoid chains and intersects carbon 2 of the sphingosine base. The bilayer interfacial plane is orthogonal to this axis. The greater inclination of the amide plane in the crystal form of hydroxystearoylcerebroside may be explained by the tighter intermolecular packing achieved in the crystal and/or the presence of the α-hydroxyl group of the acyl chain (see Pascher and Sundell, 1977). In either case, it is likely that the expanded in-plane lattice achieved in hydrated cerebroside bilayers is accommodated by moving the amide plane toward the bilayer surface (i.e., decreasing the angle of inclination). This conformational alteration would be consistent with the range of β's describing the conformation space of the L_α phase.

The good agreement between the orientation of the amide plane relative to the bilayer interface for the L_α bilayers and the crystal structure of cerebroside may suggest that the overall crystallographic conformation at the bilayer interface is preserved to a large degree in hydrated bilayers (Hamilton et. al., 1993). If this assumption holds true, then a second interesting structural feature shown in Fig. 9 *b* should be remarked upon. That is, the plane describing the initial segment of the sphingosine base between carbon 1 and carbon 5 would lie roughly orthogonal to the amide plane such that the hydroxyl group on carbon 3 orients toward the hydrated interface. Such an orientation favors intra- and intermolecular associations (e.g., hydrogen bonding) between adjacent amide groups, hydroxyl groups of the galactose and sphingosine moieties, and interfacial water. To unequivocally determine the average orientation of the (C-3)-OH group relative to the bilayer interface, similar solid-state ^{13}C-NMR methodology may be employed on cerebroside bilayers in which the carbon 3 of sphingosine is isotopically labeled.

Table 1 indicates that the average orientation of the amide plane in L_α bilayers varies slightly as the temperature is increased from 73° to 86°C. Although the change in $\Delta\sigma_R$ is small, as cholesterol concentration is increased from 0 to 50 mol%, the observed changes are evidence for the molecular interaction of cholesterol with cerebroside affecting the orientation of the amide linkage. X-ray diffraction studies show an increased bilayer periodicity as a function of cholesterol concentration at 86°C. Such a structural change at constant hydration (70 wt% H_2O) is observed as a result of the "condensation" effect, which is imparted by the sterol to lipid bilayers (Lecuyer and Dervichian, 1969) and may be consistent with molecular interaction between cerebroside and cholesterol at the amide groups, which results in a small conformational change of the carbonyl group at the interface.

TABLE 1 Chemical shift anisotropy of axially symmetric powder patterns for NPGS/CHOL L_α bilayers at the indicated temperatures

	0 mol % CHOL		50 mol % CHOL	
T (°C)	$\Delta\sigma$ (ppm)	β (degrees)	$\Delta\sigma$ (ppm)	β (degrees)
73	34.3	$37 \leq \beta \leq 53$	45.2	$34 \leq \beta \leq 47$
86	29.3	$38 \leq \beta \leq 55$	38.5	$36 \leq \beta \leq 51$

^{2}H spectra of NPGS/CHOL mixtures

The most distinctive features of the ^{2}H spectra of the NPGS/CHOL mixtures are the two clearly distinguishable spectral components observed at $T \geq 50$°C, indicating that exchange between the gel and liquid crystal phases is slow. Furthermore, the loss of spectral intensity is less than 20% over the

entire temperature range (20°C to 86°C) in which two transitions, each involving equilibrium between two lipid phases, are present. Significant spectral intensity losses are expected for intermediate exchange rates and have been observed in small molecules (Beshah et al., 1987) and when exchange occurs between two lipid phases (Skarjune and Oldfield, 1979a,b, 1982; Wittebort et al., 1981; Blume and Griffin, 1982; Ruocco et al., 1985a,b). The distinct, sharp spectral features of the ^2H spectra presented here are to be contrasted with the broadened two-component ^2H spectra observed for chain-labeled lecithins in the pretransition region (Siminovitch et al., unpublished results) and 2-[4′4′-$^2H_2$]DPPE/CHOL dispersions (Blume and Griffin, 1982). In these cases, the lateral diffusion constants are high and the domain sizes are small, leading to intermediate exchange spectra. Spectra of NPGS/CHOL mixtures are more similar to spectra of DPPE/DPPC mixtures, which show a clear superposition of gel and liquid crystal spectra in the two-phase regime (Blume et al., 1982b). Observation of the slow exchange ^2H spectra is not surprising, however, because x-ray diffraction data indicate that two separate lipid phases coexist at these temperatures: a melted NPGS/CHOL bilayer phase and an NPGS gel bilayer phase (Ruocco and Shipley, 1984). Therefore, molecular exchange between these three-dimensionally distinct phases is expected to be slow on the ^2H-NMR time scales ($\tau_c > 10^{-5}$ s).

The slow exchange between the lipid phases at 50°C $\leq T \leq$ 75°C facilitates the interpretation of these composite spectra. The ^2H spectra for chain-labeled NPGS bilayers are distinct from phospholipid spectra in that the lineshapes can be simulated using a *trans-gauche* chain isomerization hopping model (Huang et al., 1980) at temperatures below the high enthalpy (17.5 kcal/mol NPGS) transition at 82°C (Ruocco et al., 1981). Above this transition, the axially symmetric fast limit spectrum can be simulated using a nine-site jump model that includes three chain isomerization sites (*trans*, (±)-*gauche*) upon which a three-site, large-amplitude, long-axis hopping motion is superimposed (Siminovitch et al., 1985). A combination of these two motional models, one for a gel bilayer phase, the other for a melted bilayer phase, is used to describe the experimental spectra observed for the NPGS/CHOL dispersions. Representative simulations are shown in Fig. 10 (right) along with the experimental lineshapes (left) for an NPGS/CHOL dispersion containing 30 mol% CHOL. At 20°C the ^2H spectrum is simulated using a three-site (chain isomerization) hopping model where the three sites corresponding to *trans*, (+)-*gauche*, and (−)-*gauche* conformations are assigned to three lattice sites of a tetrahedron as described by Siminovitch et al. (1985). The input parameters used for the simulation are listed in the caption of Fig. 10. Note that there is no axial hop imposed for this simulated lineshape. The good spectral fit would be expected, because cholesterol forms a separate lamellar phase independently of the cerebroside gel at this temperature (Ruocco and Shipley, 1984), making the simulation analogous to a chain-labeled cerebroside spectral

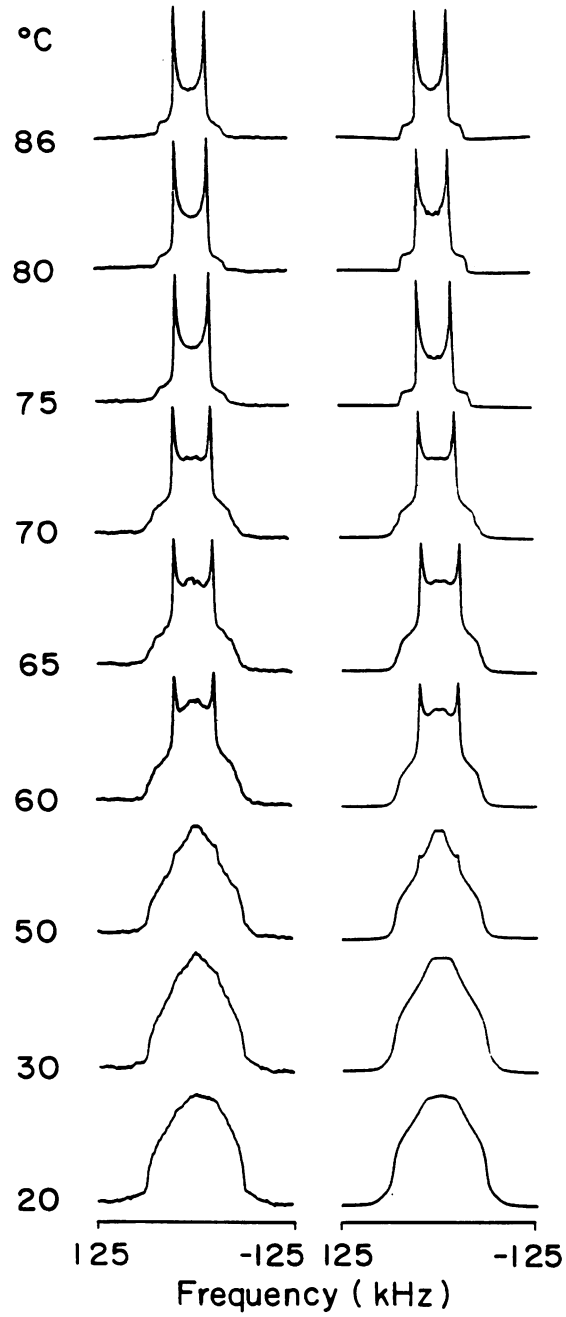

FIGURE 10 (*Left*) ^2H-NMR spectra of a dispersion of [7,7-$^2H_2$]NPGS containing 30 mol% CHOL as a function of temperature. (*Right*) Lineshape simulations obtained by an appropriately weighted summation (see Fig. 11) of an axially asymmetric gel spectral component at given temperature with an axially symmetric melted spectral component. Gel spectral parameters: (20°C) $P_1 = 0.74$, $P_2 = 0.23$, $P_3 = 0.03$, $R_{12} = 2.7 \times 10^6\ s^{-1}$, $R_{13} = 3.2 \times 10^5\ s^{-1}$; (30°C) $P_1 = 0.70$, $P_2 = 0.27$, $P_3 = 0.03$, $R_{12} = 4.0 \times 10^6\ s^{-1}$, $R_{13} = 3.2 \times 10^5\ s^{-1}$; (50°C) $P_1 = 0.64$, $P_2 = 0.30$, $P_3 = 0.06$, $R_{12} = 6.3 \times 10^6\ s^{-1}$, $R_{13} = 3.2 \times 10^5\ s^{-1}$; where R_{12} and R_{13} are the exchange rates between Sites 1 and 2 and Sites 1 and 3, respectively.

simulation (Huang et al., 1980). Corrections for power roll-off (Bloom et al., 1980) and distortions due to use of the quadrupole echo (Spiess and Sillescu, 1981) are included in the simulations. At higher temperatures (see spectra at $T =$

30°C and 50°C), narrowing of the top of the spectrum can be accounted for by a relative increase in both the (±)-*gauche* conformations and exchange rates between the conformations (see Fig. 10). At 50°C, however, the hint of a second, sharply defined component arises that can be simulated by using the nine-site jump model, which imposes fast-limit, long-axis rotation (Siminovitch et al., 1985). For the spectral lineshape at 50°C, approximately 5% of the spectral lineshape is attributed to an LG bilayer phase. We reiterate that the quadrupole splitting in the 50°C $\leq T \leq$ 65°C range is relatively constant for this phase regardless of the original stoichiometric ratio of NPGS and CHOL. We will return to the significance of this observation below. At high temperatures (i.e., $T >$ 50°C) the fraction of the LG component progressively increases (see T = 60°C, 65°C, and 70°C in Fig. 10) until a single, fast-limit, axially symmetric powder pattern is observed at 86°C. The fidelity with which the experimental lineshapes are simulated is particularly encouraging for the models employed.

Simulation of the ^{2}H spectra shown in Figs. 5 and 10, as described above, permits a determination of the fraction of the melted bilayer component over the entire temperature range. These results are summarized in Fig. 11, where the melted lipid fraction, f_m, is plotted as a function of temperature. For greater clarity and to interpret these spectral data in terms of the reported thermal behavior of NPGS/CHOL dispersions (Ruocco and Shipley, 1984), an idealized DSC trace representative of NPGS/CHOL mixtures containing <50 mol% CHOL is drawn to the temperature scale in Fig. 11.

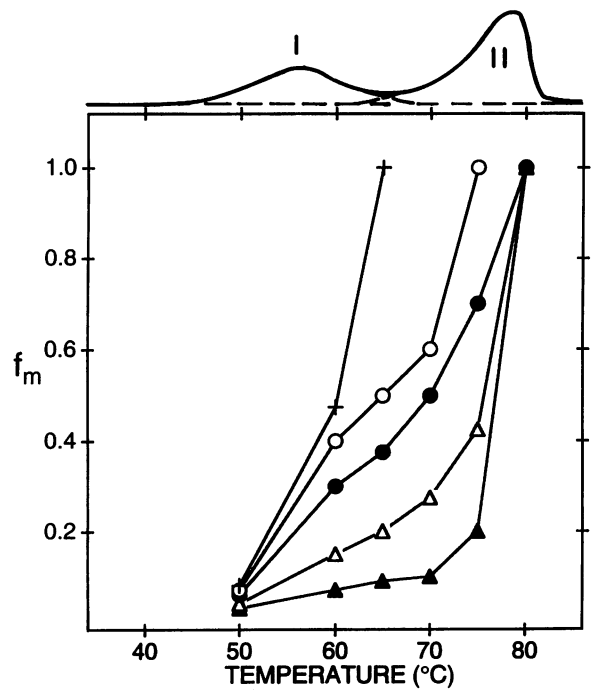

FIGURE 11 Fraction of melted lipid, f_m, obtained from lineshape simulations of the ^{2}H-NMR spectra in Fig. 5 versus temperature: (▲) 10, (△) 20, (●) 30, (○) 40, and (+) 50 mol % CHOL. Representative DSC traces are scaled to the temperature axis for NPGS/CHOL dispersions containing (*a*) 50 mol% CHOL and (*b*) <50 mol% CHOL (see also Ruocco and Shipley, 1984).

For the equimolar NPGS/CHOL dispersion a cooperative order-disorder transition (transition I) centered at approximately 56°C is observed. The presence of a relatively cooperative transition for equimolar cerebroside/CHOL dispersion is in contradistinction to the relatively low cooperative transition for equimolar phospholipid/CHOL dispersions (Huang et al., 1993). The nature of this transition can be better understood in light of the ^{2}H spectra observed for both [7,7-$^2H_2$]NPGS and 3α-$^2H_1$-CHOL in NPGS/CHOL dispersions (see Figs. 5, 7, and 8). ^{2}H spectra of 3α-$^2H_1$-CHOL, which exists in a rigid, highly ordered lamellar monohydrate crystal form (Loomis et al., 1979) at 20°C, indicate that the sterol is gradually incorporated into a more mobile lattice (i.e., the NPGS LG bilayer) as the temperature is increased over the 20°C to 53°C temperature range. Here we observe the motionally averaged ^{2}H spectrum characteristic of the LG phase. Interestingly, the intercalcation of cholesterol into the NPGS bilayer lattice begins at temperatures below or at the lower end of the relatively cooperative transition I observed by DSC. Thus, the NMR experiment appears to report the presence of this transition before it appears in the DSC trace. A similar behavior was observed in ^{13}C-NMR studies of the pretransition in PCs (Wittebort et al., 1981). This suggests that the enthalpy of transition I is partially associated with the incorporation of cholesterol into the cerebroside bilayer. Moreover, the [7,7-$^2H_2$]NPGS and the [^{13}C=O]NPGS spectra show an axially symmetric component in this temperature range, indicating that the NPGS molecule is also beginning to execute axial diffusion. With increasing temperature, the gel-like component in these spectra decreases in intensity, and the lineshape transforms to an axially symmetric component characteristic of an LG phase. Note that at 50% CHOL the chains do not significantly disorder with increasing temperature, and thus the enthalpy of transition I ($\Delta H \approx$ 9 kcal/mol) must be associated with the onset of axial diffusion. Thus, the NMR data permit a microscopic interpretation of the endotherm observed in the DSC experiment.

At lower cholesterol concentrations the low-temperature transition I is also observed. The proportion of melted cerebroside component at temperatures immediately above transition I indicate a fractional melted component similar to the molar percentage of cholesterol added to the original dispersion (Fig. 11). It is known from x-ray diffraction studies over the 0.6–50 mol% CHOL concentration range at 66°C, a temperature just above transition I, that cholesterol monohydrate reflections are no longer observed, indicating that all CHOL present has been incorporated into the NPGS bilayers. Based on the fraction of melted component in Fig. 11 and the discussion above, we conclude that CHOL intercalates into the NPGS bilayer, forming a LG phase bilayer with NPGS/CHOL in a 1:1 stoichiometry above transition I. The similarity of the residual quadrupole splitting

for NPGS/CHOL dispersions containing 10–40 mol% CHOL at temperatures in the region of transition I ($50°C \leq T \leq 65°C$) to the quadrupole splitting of the equimolar NPGS/CHOL dispersions is consistent with the existence of an equimolar NPGS/CHOL bilayer phase for these NPGS/CHOL mixtures (see Fig. 6, *a* and *b*). This is in contrast to the change in quadrupolar splitting observed on the addition of CHOL in DPPC/CHOL systems (see Fig. 6 and Huang et al., 1993).

The LG phase equimolar NPGS/CHOL bilayers coexist with an unmelted NPGS gel bilayer in NPGS/CHOL mixtures containing <50 mol% CHOL at temperatures intermediate between transitions I and II. This bilayer phase gives rise to the axially asymmetric ^2H spectrum in the two-component experimental lineshapes at T_c (transition I) $\leq T \leq T_c$ (transition II) (see $T = 60°C$, 70°C in Fig. 5, *c–e*, for example). Transition II, which is associated with the pure NPGS gel → liquid crystal transition, is asymmetrically skewed to lower temperatures in the presence of cholesterol. As this pure NPGS gel fraction melts at temperatures of >65°C, the quadrupolar splitting decreases in a divergent manner, depending on the original cholesterol concentrations in the dispersions. This divergence occurs as the residual NPGS is incorporated into the preexisting LG NPGS/CHOL bilayer phase (Fig. 6 *a*). Thus, the quadrupolar splitting is modulated by both temperature (see $\Delta\nu_Q$ for 50 mol% CHOL dispersion in Fig. 6 *a*) and CHOL concentration (see Fig. 6 *b*).

CONCLUSIONS

Although the NPGS/CHOL binary lipid system is complex, an understanding of the thermal, structural, and dynamic features of the system is an important first step to understanding the physical properties and function of myelin. First, it should be recognized that CHOL and cerebroside form the major proportion of lipid in the extracytoplasmic monolayer of the myelin sheath (Norton and Cammer, 1984; Norton, 1981). Although NPGS is not the major type of cerebroside in myelin, it is nevertheless the best characterized by a number of biophysical techniques; therefore, it is the most likely candidate for furthering our understanding of the molecular interaction between CHOL and cerebroside at the present time. The ^{13}C- and ^2H-NMR experiments described here have elucidated a number of features of the phase behavior, structure, and dynamics of this system.

The $^{13}C{=}O$ spectra of the gel state show the rigid lattice behavior of the lipid in this phase, but with increasing CHOL concentration and temperature, long-axis diffusion initiates and the spectrum narrows. A comparison of spectra obtained with cross-polarization and a Bloch decay clearly shows the coexistence of gel and liquid crystalline-like phases. Above T_c the width of the ^{13}C spectrum can be used to determine the average orientation of the amide C=O, and to a good approximation the conformation is that found in crystals. However, the observed temperature and CHOL concentration dependence indicate that this conformation changes slightly with these variables.

^2H spectra of 7,7-NPGS/CHOL mixtures share certain features with the $^{13}C{=}O$ spectra. In particular, over a rather large range of temperatures and CHOL concentrations, the spectra can be accurately simulated with a simple superposition of gel and liquid gelatin phase spectra. This indicates that exchange between these two types of lipid phases is slow on the ^2H-NMR time scale, probably because the domains are large and/or the lateral diffusion constants in the gel state domain are very slow. In addition, the ^2H spectra show that the liquid gelatin component disorders to form a component with ^2H spectra similar to those observed in liquid crystalline phases. Spectra of 3α-$^2H_2$-CHOL indicate the presence of a rigid lattice CHOL molecule that converts to a diffusing molecule at higher temperatures as it becomes part of the NPGS bilayer. The observation of these changes in the ^{13}C and ^2H spectra correlate well with the DSC results and provide a microscopic picture of the changes that occur at the thermal transition.

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